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Medium Throughput Automation of two Cytotoxicity Assays with the Eppendorf epMotion® 5075t*

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Abstract

Cell based Assays (CBA) are widely used tools in life science research and also drug development. As these assays are normally carried out in multiple plates starting from 96 well to even 1536 well formats, automation is regarded as highly useful to increase robustness and reproducibility of the obtained results. Here we demonstrate that the Eppendorf epMotion 5075t can perfectly be used for medium throughput automation of two different commercially available Cytotoxicity Assays: the Vybrant® Cytotoxicity Assay (Molecular Probes®, Life Technologies®) and CytoTox-Fluor™ (Promega®). In comparison to a manual procedure the assay robustness and data consistency could be increased by using the epMotion. For both Cytotoxicity Assay kits the entire workflow from cell seeding to the final addition of the respective assay reagents as carried out automatically on the epMotion 5075t. The workstation was equipped with the optional CleanCap module that includes air filters and UV lights, thus allowing to maintain a sufficiently clean environment for cell culture work. In total the experiments described in this study emphasize the usability of the epMotion liquid handling workstation for cell based assays.

Introduction

Drug discovery and development is a long and costly process which can be divided into four different phases: early discovery, late discovery, preclinical and clinical. About 10–15 years are necessary to develop a new medicine from the moment it is discovered to when it is available for patients. The highest cost in terms of time occurs in early and late discovery while the highest cost in terms of money is concentrated on preclinical and clinical phases. Among all potential candidates entering into the development pipeline, it has been estimated that only 1 out of 10,000 candidates really reaches the market. As costs associated with this failure considerably increase when compounds advance within this process, identifying risks in the early development phases has become essential for pharmaceutical companies [1]. There are many reasons inducing drug candidate failure during development. The molecule exclusion can be due to poor biopharmaceutical properties as solubility and stability but the two major causes of cessation are unacceptable drug's efficacy and toxicity. Historically, toxicity testing was performed just before clinical trials and generally involved animal models. To optimize drug development processes, scientists reviewed their strategy and moved toxicity tests into the early stage of development. During this phase, the number of potentially therapeutic compounds is still extremely large and cannot be tackled by methods based on animals. The emergence of cytotoxicity cell-based assays permitted to support this new developmental approach.

Cytotoxicity assays conducted during early development steps have to face various challenges. Sensitivity is crucial but the assay has also to be simple, robust, rapid and cost-effective. To allow medium or high-throughput screening (HTS), assay automation using 96-, 384- or even 1536-well formats has to be possible without affecting reproducibility and statistical significance of data [2]. The most common way to measure the cytotoxic impact of a compound is to evaluate the loss of cellular membrane integrity associated with cell death. Biomarkers generally used are constitutive enzymes released into the extracellular environment after membrane damage [3]. Assays used in this study are based on this principle and allowed to demonstrate the ability to implement a cytotoxicity cell-based assay on the Eppendorf epMotion 5075t.

Material and Methods

Methods for automated Cell-based assay
The complete workflow is programmed to process three 96-well plates in parallel and is divided into three epMotion methods. For the first two methods, the epMotion 5075t surfaces and tools are cleaned using a disinfection solution. Both UV-lights and air filters are started 15 minutes before using the system, whereas UV stopped automatically after this time span. At the end of each method, a user intervention is required to hand the plates over to downstream steps (incubation, reading).

Method 1:
Cell seeding is performed during this first protocol step. Adherent HepG2 cells are seeded into 96-well cell culture plates with transparent bottom at 30,000 cells per well. Before starting the epMotion method 1, a cell solution at the appropriate concentration (30,000 cells per 90 µL) is prepared in a sterile tube and transferred to a sterile autoclaved epMotion reservoir of 100 mL on the epMotion 5075t. Cell culture medium, used as blank, is transferred in a sterile epMotion reservoir of 30 mL. These steps need to be performed under sterile cell culture conditions. The method 1 generates the dispensing of 90 µL of cells per well in column 1 to 11 in three 96-well microplates and 90 µL of culture medium without cells in column 12 in these three plates. At the end of the program, the lid is manually replaced on the plates and the plates are placed into the CO_2 incubator at 37 °C for 24 hours.

Method 2:
The goal of the second method is to generate the concentration curve of the cytotoxic agent Ionomycin. Concentration range is between 0.03 µM and 100 µM and 16 increasing concentrations are used for inducing cell death. The concentration curve is produced by using three different dilution steps and performed in a Deepwell plate. The first dilution is performed into DMSO 99.9 % vehicle solution and the second dilution into culture medium without FBS for generating 16 ionomycin concentrations 10 times more concentrated than desired. The last dilution is performed into culture medium with 10 % FBS. Culture medium without toxic agent and DMSO being used as negative control are also added into the Deepwell Plate. At the end of this first part, the 96-well plates seeded with the cells are placed on the worktable. The second part starts with the culture medium removal from the three 96-well plates. 100 µL of each Ionomycin concentration solution is dispensed from the Deepwell plate to the 96-well plates.

The final vehicle (DMSO) concentration did not exceed 1 % and was equivalent for all Ionomycin concentrations tested. At the end of the method, the lid is manually replaced on the plates. With the cellular model used for this study (HepG2 cells), seeded plates were incubated 7 hours at 37 °C (5 % CO_2).

Method 3:
After incubation, the plates are removed from the CO_2 incubator. When the Vybrant Cytotoxicity Assay kit is used, this method includes the dispensing of 100 µL freshly prepared 2 x resazurin reaction mixture to each well of three 96-well plates. The plates are transferred to the TMX position and mixed at 500 rpm for 30 sec. At the end of this method, the lid is manually replaced on the plates and the plates are incubated at 37 °C in the incubator at humidified 5 % CO_2 atmosphere for 25 minutes. Fluorescence generated by the resazurin metabolism was read in each well (excitation at 535 nm and emission at 595 nm). When the CytoTox-Fluor Cytotoxicity Assay kit is used, this method includes the dispensing of 100 µL of CytoTox-Fluor Cytotoxicity Assay Reagent to each well of three 96-well plates. The plates are transferred to the TMX position and mixed at 500 rpm for 30 sec. With this assay, plates are incubated at room temperature for 30 minutes. Fluorescence generated by the »dead-cell protease activity« was read in each well (excitation at 485 nm and emission at 535 nm).

Those methods allow the generation of three 96-well plates. In each plate, 16 cytotoxic agent concentrations are evaluated in five replicates. Blank and negative controls are also included.

Results and Discussion

Automation of a cytotoxicity cell-based assay was initially studied by implementing the Vybrant Cytotoxicity Assay on the epMotion 5075t. This kit measures the release of the cytosolic enzyme glucose 6-phosphate dehydrogenase (G6PD) into the extracellular environment. G6PD is detected through a two-step enzymatic process that leads to the reduction of resazurin to red-fluorescent resorufin [4]. As G6PD is an abundant enzyme present in all cells and as resazurin is non-radioactive, water-soluble and stable in most culture media, a kit based on this principle represents a good candidate for automation [5]. Through the CleanCap configuration available for the Eppendorf epMotion 5075t, the workstation enclosure can be used to maintain a clean environment. For steps requiring sterile environment, epMotion 5075t surface and tools were previously cleaned with Umonium[38] disinfectant. Therefore, the complete cell-based assay protocol, including cell seeding, toxic compound dilution curve preparation and assay reagent addition, can be accomplished with the automation platform. Only plate incubation at 37 °C and data reading are carried out offline.

Comparison of manual versus automated cell-based assay performances
The automation of the Vybrant Cytotoxicity Assay with the epMotion 5075t was validated by using HepG2 cell line as cellular model system. Those cells, derived from liver tissue and commonly used in toxicology, were treated with an Ionomycin dose-response curve. Ionomycin is a bacterial ionophore used in research to affect calcium transport across cell membranes. Prolonged molecule exposure on cells results in a dose-dependent cell viability decrease and a cytotoxicity increase.

In parallel, the assay was performed manually using the same cellular model. Five replicates of each Ionomycin concentration were tested and three plates were processed in parallel. Results obtained demonstrated the capability to automate the Vybrant Cytotoxicity Assay on the epMotion 5075t (Table 1).

Table 1: Comparison of manual and automated Vybrant Cytotoxicity Assay.

		Z' factor	Global intra-plate CV (n = 5)	Global inter-plate CV (n = 3)
epMotion	P 1	0.90	6.2 %	3.90 %
	P 2	0.82	5.5 %	
	P 3	0.77	5.7 %	
Manual	P 1	0.56	13.3 %	16.8 %
	P 2	0.75	11.9 %	
	P 3	0.78	8.4 %	

To evaluate the capability of a cell-based assay to be automated, a statistical value called Z'-factor is commonly used as indicator of assay robustness. A value above 0.5 is the sign of an excellent assay quality [6]. All assays performed on the epMotion 5075t had a Z'-factor value higher than 0.75 indicating that automated assays are as robust as assays performed manually.
Besides an excellent robustness, a significant advantage offered by automating the Vybrant Cytotoxicity Assay is the decrease of the assay variability. Indeed, as intra- and inter-plate precision values show (Table 1), the assay reproducibility is highly improved by reducing human interaction. With the cellular model used in this study, the intra-plate assay is almost 2 times more reproducible when automated. For the inter-plate reproducibility, the improvement is even greater.

Implementation of Various Cytotoxicity assay kits

The number of commercially available cytotoxicity assay kits is very large. Workstations proposed as automation solution should consequently be compatible with various methods developed for measuring toxicity effect. Resazurin added for monitoring the activity of G6PD released by death cells is a permeable indicator also used to evaluate cell viability. It is a major component of viability cell-based assay kits as the CellTiter-Blue® Cell Viability Assay from Promega. Its use during a cytotoxicity assay implies that a certain amount of resazurin could be reduced by cells which are still viable. For this reason, scientists could prefer an alternative biomarker. The CytoTox-Fluor Cytotoxicity Assay kit provided by Promega measures a protease activity associated with cytotoxicity. The enzyme substrate used is a fluorogenic peptide (bis-alanyl-alanyl-phenylalanyl-rhodamine 110; bis-AAF-R110) which cannot cross the intact membrane of live cells. Consequently, signals generated can only be due to proteases released from cells that lost membrane integrity [7]. The same cellular model (HepG2 cells treated with a concentration curve of Ionomycin) was used to compare automation of both assays. Z' factor and signal-to-background ratio have been evaluated (Table 2).

Table 2: Comparison of Vybrant Cytotoxicity Assay and CytoTox-Fluor Cytotoxicity Assay on epMotion

Assay	Z' factor	S/B value
Vybrant Cytotoxicity Assay	0.70	5.00
CytoTox-Fluor Cytotoxicity Assay	0.67	7.10

As expected, the signal-to-background ratio is slightly improved when the enzyme substrate used is not able to enter into viable cells. Z' factor values clearly prove that with the epMotion 5075t, the robustness of cytotoxicity assays is guaranteed independent of the kit.

Conclusion

During the long drug discovery process, researchers rely on various parameters as viability, apoptosis and cytotoxicity to get biological information and to make their decisions. The capability to automate cell-based assays designed to monitor apoptosis [8] and cell viability [9] on the Eppendorf epMotion 5075t has already been demonstrated. In the present Application Note, we show that also cytotoxicity assays can be successfully automated with the same liquid handling workstation. Assay implementation has been evaluated with 2 commercial kits measuring the activity of an enzyme, released from cells after they were subjected to membrane damages: Vybrant Cytotoxicity Assay kit from Life Technologies and CytoTox-Fluor Cytotoxicity Assay from Promega. All assays performed had a Z'-factor value greater than 0.65, indicating the excellent robustness of the assay. Comparison to the manual method confirmed that automation with the epMotion 5075t significantly increases the assay reproducibility while reducing the hands-on time. With robustness and reproducibility being guaranteed, automating cell-based assays on the epMotion 5075t represents a perfect solution for scientists interested in a low to medium-throughput screening, no matter which cell response is of interest.

References

[1] McKim J.M. Building a tiered approach to *in vitro* predictive toxicity screening: a focus on assays with *in vivo* relevance. *Combinatorial Chemistry & High Throughput Screening* 2010; 13: 188–206.

[2] Crouch S.P.M. and Slater K.J. High-throughput cytotoxicity screening: hit and miss. *Drug Discovery Today* 2001; 6: S48–S53.

[3] Niles A.L., Moravec R.A. and Riss T.L. *In vitro* viability and cytotoxicity testing and same-well multi-parametric combinations for high throughput screening. *Current Chemicals Genomics* 2009; 3: 33–41.

[4] Vybrant® Cytotoxicity Assay kit *Product information* MP23111, Molecular Probes, Inc.

[5] Batchelor R.H. and Zhou M. Use of cellular glucose-6-phosphate dehydrogenase for cell quantification: applications in cytotoxicity and apoptosis assays. *Analytical Biochemistry* 2004; 329: 35–42.

[6] Zhang J, Chung T, Oldenburg K. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J. Biomol. Screening* 2009; 4:67–73.

[7] CytoTox-Fluor™ Cytotoxicity Assay *Technical Bulletin* #TB350, Promega Corporation.

[8] Automated Cell-based Apoptosis Assays with the Eppendorf epMotion® 5075t increases the reproducibility Eppendorf Application Note 329; www.eppendorf.com

[9] Reliable and robust automation of Cell Viability Assays with the epMotion® 5075t Application Note 344; www.eppendorf.com

Ordering information

Description	Order no. international	Order no. North America
epMotion® 5075t	5075 000 042	5075000042
epMotion® 5075t CleanCap upgrade set	5075 001 888	5075001888
Gripper	5282 000 018	960002270
TS50 pipetting tool	5280 000 010	960001010
TM50-8 pipetting tool	5280 000 215	960001044
TM1000-8 pipetting tool	5280 000 258	960001061
ReservoirRack for epMotion®	5075 754 002	960002148
CellXpert® C170i (Cell Culture Incubator)	see online	see online
epT.I.P.S.® Motion 50 µL Filter	0030 015 215	0030015215
epT.I.P.S.® Motion 1000 µL Filter	0030 015 258	0030015258
epMotion® Reservoir	0030 126 505	960051009
30 mL	0030 126 513	960051017
100 mL		
Reservoir Rack Module TC, for use in epMotion® Reservoir Racks, temperable, 4 x Safe-Lock tubes 0.5/1.5/2.0 mL	5075 799 081	960002620
Eppendorf Safe-Lock Tubes, 1.5 mL	0030 120 086	022363204
Eppendorf Deepwell Plate 96/1000 µL	0030 502 205	951032701
Waste bags	5075 752 034	5075752034

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*Developed on a predecessor model, but thanks to the migration feature, this method can easily be transferred to the newest generation of epMotion®.